Pergamon

European Journal of Cancer Vol. 31A, No. 4, pp. 453–458, 1995

0959-8049/95 $9.50+0.00

0959-8049(95)00033-X

Differentiation and Survival Influences of Growth Factors in Human Neuroblastoma

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Human neuroblastoma cell lines are established from high-stage, highly malignant tumours. Despite this and the fact that these tumours are arrested at an early, immature stage, many cell lines have the capacity to undergo neuronal differentiation under proper growth conditions. One such cell line is the noradrenergic SH-SY5Y cell line. These cells can be induced to mature by a variety of modalities, resulting in different mature phenotypes. The use of this cell system as a model to study the stem cell character of neuroblastoma is reviewed and discussed. In particular, we focus on growth factor dependencies in the SH-SY5Y system, and compare that to the normal situation, i.e. growth factor control of sympathetic neuronal and neuroendocrine differentiation during human and rat embyrogenesis.

Key words: neuroblastoma, neurotrophins, insulin-like growth factors, fibroblast growth factors, differentiation, apoptosis, sympathetic nervous system, sympathoadrenal

Eur J Cancer, Vol. 31A, No. 4, pp. 453–458, 1995

INTRODUCTION

DISSEMINATED NEUROBLASTOMA is a childhood tumour for which treatment is unsuccessful in the majority of patients, despite the introduction of very intense and demanding treatment regimens. However, neuroblastoma as a group is a disease with at least two facets. Its heterogeneity in terms of patient outcome can be largely resolved if neuroblastomas are categorised according to tumour site or age at diagnosis. Children under 1.5 years of age generally present with an extra-adrenal tumour and a favourable outcome, while children in the older age group usually have an adrenal tumour and a poor outcome [1]. Neuroblastoma might thus be comprised of different but closely related diseases, and in tumour biological terms, these observations suggest that neuroblastomas are derived from more than one sympathetic lineage. If such is the case, the clinical heterogeneity should be demonstrable at the biochemical level. This is indeed the situation, for example, we have found that IGF-II (insulin-like growth factor II) expression is mainly confined to extra-adrenal, low-stage tumours, where IGF-II marks neuroendocrine differention [2]. Although the treatment of patients with highly malignant tumours is not improved by knowing that their tumours differ from low-stage tumours in their lineage, such insight helps to focus future efforts. Thus, we believe that biological characterisation of high-stage tumours is important, and that such knowledge will be the basis for establishing future successful treatment protocols for neuroblastoma.

To address this issue, we have followed principally two different lines of investigation. One uses population-based tumour material, which is characterised in terms of clinical, tumour biological and phenotypical parameters, with the aim of identifying clinical sub-groups of tumours [1–7]. The other line of investigation focuses mainly upon growth and differentiation aspects of cultured neuroblastoma cells, which is the main subject of this review. In both projects, access to fixed human fetal tissue, fetal and postnatal rat material, as well as primary cultures of rat sympathoadrenal cell derivatives, has been essential since these tissues and cell culture systems serve as references of normal sympathetic nervous system development.

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CELL LINES AS MODEL SYSTEMS FOR HIGH-STAGE NEUROBLASTOMAS

There are conceptual as well as practical rationales of using established cell lines to study the biology of high-stage neuroblastomas. Firstly, all neuroblastoma cell lines are established from highly malignant tumours. Secondly, many of these cell lines can be grown under conditions which induce differentiation as exemplified by SH-SY5Y, SMS-KCNR and LA-N-5 cells (reviewed in refs [8] and [9]). Taken together, these findings show that highly malignant neuroblastoma cells are arrested in their differentiation, and that at least some high-stage tumours have an intact and inducible capacity to mature *in vitro*. The comparatively low incidence of neuroblastoma (10–15 cases a year in Sweden) makes it difficult to perform studies on more than a few primary cultured tumours at a time, not to mention problems caused by the biochemical and cell biological heterogeneity of such material. For practical reasons, we have, therefore, worked with neuroblastoma cell lines that have the capability of undergoing neuronal maturation. Besides the usefulness of obtaining large cell numbers, cell lines also offer populations of clonal cells that divide or differentiate under controlled conditions.

THE SH-SY5Y CELL SYSTEM

With few exceptions, neuroblastoma cell lines are established from tumours with an amplified *MYCN* gene. The SK-N-SH

cell line is one exception to this rule, and since this is the parental cell line of the noradrenergic subclone, SH-SY5Y, the subclone also lacks *MYCN* gene amplification [4]. The SK-N-SH cell line was established from a bone marrow aspirate of a thoracic, catecholamine secreting, neuroblastoma of a 4-year-old girl [10]. The cells are near diploid [8, 10] and have noradrenergic features, although two distinct populations of cells have been identified. In a series of elegant experiments, Biedler and coworkers have shown that SK-N-SH as well as other neuroblastoma cell lines can switch between a neuronal (N) and a surface-adherent (S) epitheloid phenotype [11]. Of the N-type subclones derived from SK-N-SH cells, SH-SY5Y has been the most studied. Our *in vitro* systems are based primarily on this subclone, as differentiation can be induced by several different defined culture conditions, driving these cells towards mature phenotypes that vary depending on the inducing agent used, which indicates that SH-SY5Y cells are multipotent. Two clearly distinct phenotypes can be induced, one by the phorbolester, 12-O-tetradecanoyl-13-phorbol acetate (TPA), in combination with serum or growth factors, and another by retinoic acid (RA).

Phorbolester-induced differentiation

SH-SY5Y cells treated with nanomolar concentrations of TPA cease to proliferate while differentiating morphologically, i.e. sending out growth cone terminated neurites which have frequent varicosities [12]. The cells acquire a neuronal phenotype, as judged by biochemical and functional markers. Expression of neuron specific enolase (NSE) and the axonal growth cone protein GAP43 increases, with GAP43 protein enriched in isolated SH-SY5Y growth cones [13, 14]. At the neurotransmitter level, the noradrenalin concentration and the expression of neuropeptide tyrosine (NPY) and tyrosine hydroxylase (the rate-limiting enzyme in catecholamine synthesis) increase dramatically [12–16]. Furthermore, dense core vesicles and vesicle proteins, such as synaptophysin, synaptic vesicle protein II and secretogranin II, increase in concentration, while another vesicle protein, chromogranin A, decreases [12, 17]. The SH-SY5Y cells express acetylcholine receptors, and differentiated cells have an increased resting membrane potential [18]. Acetylcholine causes a depolarisation of these cells, whereby stored noradrenalin is released [18]. The expression of GAP43, the neurotransmitter profile (noradrenalin, NPY), the acetylcholine-evoked neurotransmitter release, and the decrease in chromogranin A expression seen with differentiation, strongly suggest that TPA-treated SH-SY5Y cells mature along a sympathetic neuronal differentiation lineage.

The TPA-induced differentiation suggests that protein kinase C (PRKC) is mechanistically involved in the SH-SY5Y differentiation process. There has been a great deal of controversy regarding the role of PRKC, and whether differentiation is driven by down-regulation and/or activation of PRKC. Early reports showed that PRKC is membrane-translocated and partially down-regulated in TPA-differentiated SH-SY5Y cells [19], while later studies, using isoform specific antisera, demonstrated that morphological differentiation could be induced by microinjection of these antisera [20]. Both findings have been interpreted in favour of PRKC down-regulation as an important differentiation initiating event. However, development of the neuronal phenotype is concentration-dependent, with an optimal effect obtained around 16 nM TPA, and almost no effect at 1.6 μM [12, 21]. Treatment with 16 nM TPA causes modest down-regulation of PRKCA, but not PRKCE or PRKCZ, and the total PRKC activity remains elevated over several days as measured by membrane translocation of the kinase and phosphorylation of the endogeneous PRKC substrate, MARCKS [22, 23]. 1.6 μM TPA causes an almost complete down-regulation of PRKCA, similar to the effect of bryostatins, while both treatments induce only a weak and incomplete initial differentiation response, suggesting that PRKC must be active for more mature differentiated phenotype to develop [22–24]. Also consistent with this conclusion is the increased and sustained AP-1 transcription activity in SH-SY5Y cells treated with 16 nM TPA [25]. In contrast to 16 nM TPA-differentiated cells, cultures treated with 1.6 μM TPA retain their proliferative capacity (see below).

RA-induced differentiation

RA treatment results in growth inhibited, adherent cells which have long neuritic cell processes [26]. A moderate increase in NSE activity, as well as GAP43 expression [26, 27] lends support to the conclusion that RA-treated cells mature neuronally. However, several observations suggest that these cells do not differentiate sympathetically. RA fails to induce NPY or catecholamine synthesis, and blocks TPA-induced noradrenalin production [26]. However, RA-treated cells develop a slightly higher choline acetyltransferase activity suggesting that they switch to a cholinergic phenotype [28]. Recently it was shown that RA treatment induces *TRKB* expression [29], the brain-derived neurotrophin (BDNF) receptor, which is not expressed in human fetal sympathetic neurons [7]. However *TRKB* is expresed in chromaffin cells of the paraganglia and in small intensely fluorescent (SIF) cells [7], which could suggest that RA-treated *TRKB* expressing cells, that respond to BDNF by morphological differentiation [29], mature along a sympathetic chromaffin lineage. Phenotypic characterisation of these cells will indicate the accuracy of this hypothesis.

Protein kinase C and growth factor-induced differentiation

As mentioned above, TPA-differentiated cells are growth inhibited with down-regulated *MYC* expression, and when restimulated with fresh medium containing fetal calf serum, ornithine decarboxylase activity, *CMYC* expression, and thymidine incorporation are not induced [30]. Nanomolar concentrations of insulin, IGF-I and IGF-II are mitogens for the nondifferentiated SH-SY5Y cells, and these cells express receptors for insulin, IGF-I and IGF-II [30, 31]. However, once the cells have differentiated after TPA treatment, none of these three factors can stimulate cell division, but despite that functional receptors for insulin and IGF-I/IGF-II persist as shown immunologically and by binding studies [30, 31]. The functionality of the receptors was demonstrated by ligand-induced receptor autophosphorylation, actin reorganisation and *CFOS* induction [31]. Thus, the growth control in differentiated SH-SY5Y cells is, at least in part, the result of an uncoupling of growth factor receptors from mitogenic signal transducing pathways.

The fact that post-mitotic neurons in brain and differentiated SH-SY5Y cells express receptors for insulin and IGF-I/IGF-II, suggested that these receptors and factors regulate some biological activity in mature neurons. We recently found that the SH-SY5Y cells differentiate poorly with TPA under serum- and growth factor-free conditions [15]. However, when the defined medium was supplemented with IGF-I or IGF-II, TPA treatment induced neuronal differentiation similar to that induced in the presence of serum [15]. Since the TPA-treated cells down-regulate *CMYC*, both in the absence and presence of

IGF-I, it was concluded that growth control in the differentiated cells is a TPA, and presumably a PRKC-mediated effect, and that development of the neuronal phenotype requires additional serum factors such as IGF-I or IGF-II [15]. In follow-up studies, a number of growth factors, platelet derived growth factors (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) were shown to act synergistically with TPA in inducing differentiation, while alone they are mitogens for SH-SY5Y cells [30]. Since each of these growth factors can activate PRKC, although the responses are weaker, more transient, and possibly involve a different PRKC repertoire compared with TPA, different combinations of growth factors were tested for their capability to induce maturation of SH-SY5Y cells. In particular, a combination of IGF-I and bFGF induces morphological and biochemical differentiation [32], while other combinations are less or non-efficient. The bFGF/IGF-I treated cells showed no down-regulation of PRKC proteins, but had a sustained increased kinase activation, supporting the idea that transition towards a sympathetic neuronal phenotype requires functional PRKC [22, 23, 31]. These results also show that SH-SY5Y cells can mature without down-regulating PRKC. However, bFGF/IGF-I treatment is not accompanied by inhibited growth, suggesting mechanistic differences between TPA/growth factor and bFGF/IGF-1 induced differentiation. In the latter cells, *CMYC* is not down-regulated, which indicates a putative impaired growth control step that can be rescued by low, but not high, concentrations of TPA. We do not have the molecular explanation for this TPA effect, but presumably it involves a strong and sustained activation of several PRKC isoforms.

Do neuroblastoma cell lines respond to nerve growth factor (NGF)?

One notable observation during these studies was the inability of NGF to trigger differentiation of the SH-SY5Y cells, either in the absence or presence of TPA [32] despite the fact that they express the high affinity NGF receptor pp140^{ctrk}, and the low affinity neurotrophin receptor p75 (p75^{LNGFR}) [33–35]. Since expression of *CFOS* could not be induced, as is also the case for several other *CTRK* expressing neuroblastoma cell lines [33, 34], and since TPA-differentiated SH-SY5Y cells have increased *CTRK* expression without becoming NGF responsive [33], one hypothesis to explain these data would be that neuroblastoma cells of high stage tumours express a defective pp140^{ctrk}. To test that, we transiently expressed an exogenous *CTRK* gene into SH-SY5Y and LA-N-5 cells, which restored NGF responsiveness as judged by NGF-induced neurite outgrowth [33]. Stable SH-SY5Y transfections also generated cells that differentiate morphologically and functionally in response to NGF, although the pp140^{ctrk} levels in isolated cell clones were not much higher than in wild type cells [33]. In addition, the TPA-differentiated wild type SH-SY5Y cells had a *CTRK* expression level comparable with that of many NGF-responding *CTRK* transfected clones [33]. Taken together, these findings are consistent with impairment of endogenous pp140^{ctrk} signalling in at least some neuroblastomas. However, there are several reports showing that SH-SY5Y cells treated with mitogenic blockers, such as aphidicolin, differentiate morphologically in response to NGF [36–38], suggesting that growth inhibited wild type SH-SY5Y cells have an intact capability to differentiate via a pp140^{ctrk} pathway. It is not clear what phenotype the aphidicholin/NGF-treated cells acquire as the cells have not been characterised in any detail. For instance, the *CTRK* expression level is not known, and such data and future sequence analyses of the *CTRK* gene in these cells will eventually show whether there is an impairment due to mutations in the receptor gene or if the inability to differentiate in response to NGF merely reflects pp140^{ctrk} levels. Whatever the outcome of these experiments, these neuroblastoma cells clearly have defects, other than those that are *CTRK* related, contributing to the malignant phenotype. In addition, NGF-treated *CTRK* transfectants continue to proliferate [33], showing that differentiation and growth control are not tightly linked, and that these neuroblastoma cells have a deregulated mechanism for growth control.

With the stable SH-SY5Y *CTRK* transfectants, an *in vitro* human cell system to study NGF-evoked intracellular events has been generated for use in future studies. Interestingly, the most mature phenotype was achieved when these clones were differentiated by a cocktail of factors, i.e. NGF, IGF-I and bFGF [33]. These are the three factors required for cultured sympathoadrenal stem cells from rat cervical ganglia to differentiate into sympathetic neurons [39], and we conclude that the growth factor responses of the generated *CTRK* transfectants are those expected for a sympathetically differentiating neuroblast.

GROWTH FACTOR INFLUENCES IN NEUROBLASTOMA TUMOURS

The identification of specific growth factors and corresponding receptors with putative roles during tumorigenesis and growth of neuroblastoma is a virtually unexplored research area. In cell lines, a number of different growth factors are mitogens, as exemplified above, and consequently the corresponding receptors are expressed. In an interesting study by El-Badry and associates [40], it was shown that of 8 neuroblastomas, 2 expressed IGF-II, and that all tumours expressed the IGF-I receptor. They further demonstrated that one cell line, SK-N-AS, expressed and produced IGF-II, and that IGF-II-stimulated growth could be blocked by anti-IGF-I receptor antibodies, strongly suggesting that IGF-II is an autocrine growth factor for these cells [40]. This study did not indicate whether differences in clinical stage or outcome existed between the IGF-II positive and negative tumours. However, from another perspective, Hedborg and associates [2] analysed IGF-II expression in neuroblastoma tumour material and made references to clinical parameters. The incentive for that study stems from the observed overgrowth of children with Beckwith-Wiedemann syndrome (BWS), including an increased risk of embryonal tumour formation, such as low malignant extra-adrenal infant neuroblastomas, and that involvement of an increased gene dosage and/or expression of IGF-II has been implicated in this disease ([41] and refs therein). There is a nearly perfect correlation between IGF-II expression in normal human fetal tissues, and the cell type-specific pattern of overgrowth and tumour formation of the BWS, lending further support to the hypothesis that overproduction of IGF-II might, in fact, be involved in the genesis of these tumours, including extra-adrenal neuroblastomas [41]. Using *in situ* hybridisation to demonstrate IGF-II expression at the cellular level in the fetal sympathetic nervous system and in neuroblastomas, it was shown that IGF-II positivity was specific for extra-adrenal chromaffin cells during normal development, and in neuroblastoma it was essentially confined to extra-adrenal, low-stage, tumours [2]. The spatial arrangements of the IGF-II positive cells were most remarkable as they formed ring-like structures. Closer analyses revealed that IGF-II expression marks differentiated cells with neuroendocrine characteristics, and that they are found within tumour lobular structures

surrounded by blood vessels and stromal cells [2]. The IGF-II positive cells were Ki-67 negative, and thus non-proliferating. Dividing immature cells were found adjacent to the stroma and blood vessels, while the IGF-II positive cells were positioned away from the stroma in a central zone of each tumour lobule, surrounding a core of apototic cells and cell remnants [2, 6]. Based on these *in vivo* findings, it is difficult to envisage an autocrine growth promoting function for IGF-II in neuroblastoma, or a role as a differentiation promoting factor, since that does not account for the BWS-hyperplasia and neoplasia of cell types with an abundant IGF-II expression during normal development. An interpretation more consistent with available data would be that IGF-II serves as a survival factor preventing programmed cell death. Such a function would account for both the pattern of hyperplasia seen in BWS, and the presence of viable, non-proliferative, IGF-II positive cells close to IGF-II negative cells undergoing programmed cell death in extra-adrenal neuroblastomas. The autocrine mitogenic IGF-II loop demonstrated in cultured neuroblastoma cells [40] might represent an exception among neuroblastomas, or could be an *in vitro* artefact, which in such a case is not the first example of cultured cells switching their growth factor production as they are propagated *in vitro*.

Several recent reports have demonstrated that the *CTRK* and *p75* low affinity NGF receptor genes are expressed in most neuroblastomas [42–45]. For both receptors, the expression is highest in low-stage tumours, and in a few high-stage tumours, expression was barely or not detectable. Primary cultured low-stage tumour cells were shown to be dependent on NGF for survival, and they differentiated morphologically in the presence of NGF [42]. In this study, no primary cultures of high-stage tumours were studied, but presumably such cells will not respond to NGF. In an immunocytochemical study of pp140^{ctrk}, pp145^{trkB}, and pp140^{trkC} expression in neuroblastoma, we confirmed that low-stage tumours had the highest pp140^{ctrk} immunoreactivity, and that high pp140^{ctrk} levels correlated positively with favourable outcome [7]. We also found that many low-stage tumours with lobular architecture contained regions of differentiated cells with high pp140^{ctrk} immunoreactivity closely opposed to areas of apoptotic or dead cells [6, 7]. This could suggest a causal relationship between NGF dependence, NGF shortage in central parts of the tumour lobule, and apoptotic cell death in these regions of the tumour. The clinical importance of these NGF receptor studies is firstly that *CTRK* mRNA and pp140^{ctrk} expression levels provide prognostic information, and secondly, that straightforward NGF treatment will most likely not affect highly malignant neuroblastomas, and thus not improve outcome for this patient group. It is possible, however, that blockade of NGF receptors or NGF-induced signalling in low-stage tumours might be an elegant way of treatment by inducing cell death.

pp140^{trkC} immunoreactivity is also frequently seen in neuroblastomas, suggesting that NT3/NT4 might influence growth, differentiation, and survival, although no statistically significant correlation between pp140^{trkC} levels and favourable outcome could be demonstrated [7]. Finally, based on northern blot analyses, *TRKB* was shown to be expressed in most high stage tumours with *MYCN* amplification, as well as in most ganglioneuromas and ganglioneuroblastomas [46]. Immunohistochemical analysis revealed that pp145^{trkB} immunoreactivity was rarely present in proper tumour cells, but was instead localised to stromal cells [7], a finding in keeping with the reported ganglioneuroblastoma positivity based on mRNA data

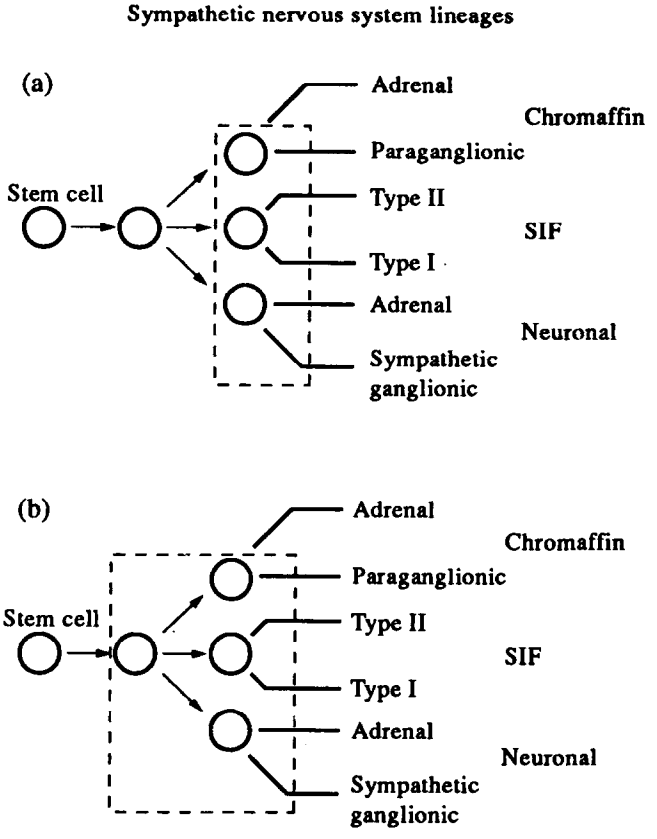

Figure 1. Neuronal and neuroendocrine cell lineages of the sympathetic nervous system, and their putative relation to neuroblastoma. The three major differentiation lineages, neuronal, SIF (small intensely fluorescent) and chromaffin, are shown in the figure [2, 49]. Each gives rise to at least two cell types, which for each lineage differ in location [49] and phenotype, as exemplified by phenylethanolamine-N-methyl transferase (PNMT) negative paraganglionic and the PNMT positive adrenal chromaffin cells. The boxes enclose differentiation stages that neuroblastomas may derive from. The chromaffin adrenal differentiation pathway is excluded, since neuroblastomas do not express PNMT [5], the key marker enzyme of chromaffin adrenal cells. (a) In the first model, neuroblastomas are derived from a set of progenitor cells with defined lineage and the patterns of gene expression and phenotypes found in neuroblastoma reflect the lineage origin of the initially transformed cell. (b) In the second model, in addition to derivation from defined lineages, neuroblastomas derive from immature progenitor cells with capability to differentiate into more than one sympathetic lineage. The model also suggests that transformed cells at early stages, on some occasions, spontaneously differentiate along one of the lineages.

[46]. In tumour cells proper, pp145^{trkB} immunoreactivity was seen only in one case out of 30 [7]. The discrepancy regarding the high-stage tumour data between the two studies might be a reflection of the size of the material sampling. As suggested by Nakagawara and associates [46], the BDNF pp145^{trkB} pathway appears to be important for growth and differentiation of *MYCN*-amplified neuroblastomas, and is, therefore, a candidate target for novel clinical treatment protocols. As discussed above, *TRKB* expression in high stage tumours is difficult to understand in terms of cell lineage occupation, since fetal sympathetic ganglia do not express significant amounts of pp145^{trkB} [7]. Instead, the overall low pp145^{trkB} immunoreactivity in the tumours in our study [7] is consistent with the presumed neuronal sympathetic derivation of neuroblastomas. We know that most neuroblastomas with an amplified *MYCN* gene are arrested at early stages of development [1]. *TRKB* expression

might thus reflect such early stages, although we have not detected pp145^{trkB} immunoreactivity in sympathetic neuroblasts, not even in the earliest fetal stages (week 7.5) available to us [7].

ARE ALL NEUROBLASTOMAS DERIVED FROM THE SAME PROGENITOR CELL?

The phenotypical heterogeneity of neuroblastomas, as indicated by both neuronal and neuroendocrine traits, could indicate that more than one cell type gives rise to this tumour (Figure 1a). Alternatively, it might reflect pluripotency of a progenitor cell in common, a progenitor that has self-renewal capacity but most likely is not a neural crest stem cell but rather a cell with intermediate characteristics (Figure 1b). The latter model is consistent with cell line data, which show that several cultured neuroblastomas can mature along more than one lineage, as exemplified by the SH-SY5Y cells described here, and by the SMS-KCNR cells described by Thiele and coworkers [9, 47]. The interconversion of neuroblastic neuroblastoma cells (N-type) into a surface adherent phenotype (S-cells) [11] could also be an example of pluripotency, although the normal counterpart to S-cells has not been identified or shown to exist. With regard to this, one should remember that stromal or Schwann cells of low-stage neuroblastomas and ganglioneuromas are diploid, and thus not the result of neuroblastoma cells converting into stromal cell phenotypes [48]. Our finding that many low-stage neuroblastomas show neuroendocrine characteristics supports the first model [2], but the gradient of differentiating cells seen in the lobular structures of these tumours might reflect a conversion of immature cells of a neuronal lineage into neuroendocrine cells, and thus be an example of pluripotency of neuroblastoma cells. Nevertheless, present data do not distinguish between these two models, but if the cellular origin(s) of the various clinical forms of neuroblastoma is understood, new treatment strategies might be formulated, based on growth factor and hormone-induced differentiation or programmed cell death.

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Pergamon

European Journal of Cancer Vol. 31A, No. 4, pp. 458–463, 1995

0959-8049/95 $9.50+0.00

0959-8049(95)00006-2

Proliferation and Apoptosis in Neuroblastoma: Subdividing the Mitosis-karyorrhexis Index

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The Shimada classification is a frequently used, histopathological classification system for neuroblastoma tumours. Tumours are classified as prognostically favourable or unfavourable based upon stroma content, degree of neuroblastic maturation and patient age at diagnosis. The mitosis-karyorrhexis index is introduced in this classification system, as the cellular density sum of mitotic and karyorrhectic cells in the tumour. The biological nature of karyorrhectic cells is uncertain, but a high mitosis-karyorrhexis index in stroma-poor tumours is an indicator of poor prognosis. In this study, neuroblastoma tumours were analysed for cell proliferation, using antiproliferating cell nuclear antigen (PCNA) immunohistochemistry, and apoptosis, by morphology and *in situ* end-labelling of fragmented DNA. The karyorrhectic cells described in the Shimada classification were shown to be either proliferating or undergoing apoptosis. It is further shown that a high cellular density of proliferating cells correlates with poor prognosis, whereas a high density of apoptosis, in contrast, indicates favourable outcome.

Key words: mitosis-karyorrhexis index, programmed cell death, apoptosis, neuroblastoma
Eur J Cancer, Vol. 31A, No. 4, pp. 458–463, 1995